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Age Distribution of Antibodies to Human Papillomavirus in Children, Women with Cervical Intraepithelial Neoplasia and Blood Donors from South Africa

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Sera from 95 women with cervical intraepithelial neoplasia (CIN), 95 age-matched female blood donors, and 155 children aged between 1 and 12 years were tested by enzyme-linked immunosorbent assay (ELISA) for levels of serum IgG to three human papillomavirus (HPV) peptides (HPV-16 E2 [E2–16], HPV-18 E2 (E2-18], HPV-16 L1 [L1-16]), as well as HPV-16 virus-like particles (VLP-16) and bovine papillomavirus type 1 virus-like particles (BPV-VLP). In the adult group antibodies to E2-16 and VLP-16 were significantly associated with CIN when compared to the blood donor controls ($P = .039$ and $P = .002$, respectively). In women with CIN there was an increase in seropositivity to E2-16 and a decrease in seropositivity to VLP-16 with age. Antibodies to HPV-16 E2 could therefore be an important marker of CIN in women over 40 years of age, whereas antibodies to VLP-16 could be a marker for CIN in younger women. There was no correlation with CIN and antibodies to E2-18, L1-16, and BPV-VLP. In the children's sera antibodies were detected to E2-16 (44.5%), E2-18 (18.7%), L1-16 (20%), VLP-16 (4.5%), and BPV-VLP (5.1%). Between the ages of 3 and 12 years the prevalence of antibodies to E2-16 decreased with age. The presence of antibodies to HPV-16 in young children indicated infection with either HPV-16 or a related virus. HPV DNA isolation from children could help resolve this question. ***J Med Virol 51:126–131, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: ELISA; CIN; serology; HPV-16; HPV-18; virus-like particles

INTRODUCTION

Epidemiological and molecular evidence indicates that specific types of human papillomavirus (HPV) play a significant role in the development of cervical intraepithelial neoplasia (CIN) and cervical cancer [Schiffman, 1992]. South Africa has an extremely high incidence of cancer of the cervix and at least 4.7% of black women will develop cancer of the cervix if the present trend continues [Sitas and Pacella, 1994]. Cervical screening programmes are inadequate, and a good HPV vaccine would have a major impact on the incidence of cervical cancer in this population. Human papillomavirus type 16 (HPV-16) has been demonstrated to be associated with 46% of cervical cancers and 21% of CIN grade 3s (CIN III) in Cape Town [Williamson et al. 1989; Williamson et al., 1994]. In these studies the incidence of HPV 18 was low and was detected in only 1.5% of cervical cancers and 1% of CIN III biopsies. However, in a study of Cape Town women with normal cervical cytology HPV-18 was the predominant HPV type detected [Ramesar et al., 1996]. No studies have been carried out on the prevalence of antibodies to HPV antigens in South Africa.

In recent years serological assays for HPV have been developed based on fusion proteins, peptides virions, and virus-like particles (VLPs) [Bonnez et al., 1991; Cason et al., 1992; Dillner, 1994; Galloway, 1994]. Most studies have attempted to link the seropositivity to specific HPV antigens with the disease status of the patient [as reviewed by Dillner, 1994; Schiller and Roden, 1995]. Recent studies have demonstrated that antibodies to VLPs can be used as markers of infection [Rose et al., 1993, 1994a; Schiller and Roden, 1995] as well as disease [Nonnenmacher et al., 1995; Dillner et al., 1995]. Antibodies to HPV-16 VLPs (VLP-16) and E2-derived peptide 245 (E2-16) have also been used to detect HPV seroconversions [Wikstrom et al., 1995]. Although anti-

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bodies to HPV can be an indication of past infection, they are apparently short-lived in the case of transient infections [Wikstrom et al., 1995]. Information on the prevalence of HPV 16 infection is needed when considering the feasibility of HPV-16 L1–based vaccines.

In this study three groups of people were tested for serum IgG levels to an HPV-16 L1 peptide [Cason et al., 1992], VLP-16 [Rose et al., 1994b], an HPV-16 E2 peptide, an HPV-18 E2 peptide [Dillner et al., 1994], and bovine papilloma virus type 1 (BPV-1) VLPs [Ghim, 1996]. This study provides novel information on the prevalence and age distribution of HPV antibodies in children, women with cervical disease, and age-matched female blood donors. It also analyses serological markers for CIN according to the age of the patients.

MATERIALS AND METHODS

Serum Samples

Intermittently sequential serum samples were drawn from 95 patients with CIN who attended a colposcopy clinic at Groote Schuur Hospital, Cape Town, South Africa, during 1994. Biopsies of 74 patients showed CIN III, 15 patients showed CIN II, and six patients showed CIN I by standard histological criteria. Age-matched controls were selected from female Western Province Blood Transfusion Service donors within age ranges of 5 years. The number of patients and controls per age group were five aged 21–25 years, 19 aged 26–30 years, 29 aged 31–35 years, 16 aged 36–40 years, 12 aged 41–45 years, six aged 46–50 years, and eight older than 50 years. One hundred fifty-five children's sera were obtained from patients at Red Cross Children's Hospital, Rondebosch, Cape Town and from private pathologists in Cape Town. These sera had been submitted to the laboratories for routine diagnostic tests unrelated to HPV. The numbers of children per age group were two aged 1 year, 23 aged 2 years, 31 aged 3–4 years, 24 aged 5–6 years, 27 aged 7–8 years, 28 aged 9–10 years, and 28 aged 11–12 years. There were 76 female children, 71 male. The sex of eight of the children was not recorded.

HPV Antigens

Peptides. The three peptides selected for this study was E2-245-16 (HKSAIVTLTYDSEWQRDQC) [Dillner et al., 1989], E2-245-18 (EKTGILTVTYHSETQRTKC) [Dillner et al., 1994], and L1-16 (LKAKPKFTLGKRKATPTTS) [Cason et al., 1992]. These were designated E2-16, E2-18, and L1-16, respectively. The peptides were synthesised by Genosys, Cambridge, England.

VLP production and purification. For the production of VLP-16, HPV-$16_{Rochester}$ L1 was cloned using methods described previously [Rose et al., 1993] from viral DNA recovered from experimentally induced HPV-16 lesions propagated in the severe combined immunodeficiency (SCID) mouse [Bonnez, personal communication]. Recombinant baculoviruses were constructed as described previously [Rose et al., 1990 and 1993]. Recombinant baculovirus vector expressing BPV-1 L1(AcBPV1L1) was generously provided by S.-J. Ghim and A.B. Jenson (Georgetown University, Washington, DC). VLPs were produced by infecting cultures of *Trichoplusia ni* insect cells (Hi5™ cells, Invitrogen, San Diego, CA), which were propagated in 100-ml shake cultures (1-litre flasks, 125 rpm, 27°C) in ExCell 400 serum-free medium (JRH Biosciences). Cells, at a density of 2×10^6 cell/ml, were infected at a multiplicity of infection of 3, and infections were allowed to proceed for 72 hr at 27°C. Following this, infected cells were pelleted at 800*g* and resuspended in 3 ml of buffer N [Phosphate buffered saline (PBS); 0.5 M NaCl; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 mM Aprotinin; 1 mM leupeptin]. Resuspended cells were sonicated on ice (4×5 sec bursts), sonicates were diluted with PBS, and caesium chloride was added to a final concentration of 0.4 g/ml. Following high-speed centrifugation (100,000*g*, 18 hr, 15°C), VLP bands were removed by syringe and dialysed against buffer N (18 hr, 4°C). Total protein concentrations were determined using a commercial assay (MicroBCA, Pierce Biochemicals, Inc., Rockford IL, USA).

Enzyme-Linked Immunoabsorbent Assays (ELISA) Techniques

The peptide ELISAs were carried out as described by Dillner [1990] with some modifications. Microtitre plates (Nunc-Immuno, MaxiSorp, Roskinde, Denmark) were coated overnight at 4°C with 100 μl of the appropriate peptide antigen in 10 mM carbonate-bicarbonate buffer, pH 9.6. The peptides were diluted to a concentration of 20 μg/ml giving a coating quantity of 2 μg/well. Unbound antigen was removed, and potential free binding sites were blocked with a 1% milk powder in PBS-T (PBS + 0.05% Tween) for 60 min at 37°C. Plates were washed 3× with PBS-T, 100 μl of serum sample (diluted 1:20 in 1% milk powder in PBS-T) was added to each well and incubated at 37°C for 60 min. Wells were washed 3× with PBS-T, and then 100 μl horseradish peroxidase–conjugated rabbit–anti-human IgG (Dako, Sweden), diluted 1:6,000, was added and incubated for a further 60 min at 37°C. The wells were washed 3×, and 100 μl 1,2-phenylenediamine (Dako, Sweden) in 0.1 M citric acid-phosphate buffer, pH 5 (diluted according to the manufactures instructions), was added with 0.006% hydrogen peroxide. Plates were left at room temperature in the dark. The colour reaction was stopped after 30 min by the addition of 100 μl of 2 M sulphuric acid, and absorbence was read at 429 nm (A_{492}) on an Anthos 2001 plate reader (Anthos Labtec Instruments, Salzberg/Itzling, Austria). For each serum the difference in optical density (dOD) was calculated (OD of the mean of duplicate antigen-coated wells minus the mean of the duplicate OD on wells coated with PBS-T buffer only).

For the VLP ELISAs [Rose et al., 1994a] the antigen was diluted to 10 μg/ml in cold PBS and incubated on plates, 100 μl/well, at 4°C, overnight. The VLPs were removed, and the plates were blocked with 1% milk powder in PBS for 120 min at room temperature. After washing wells 3× with PBS, serum was added diluted

TABLE I. Prevalence of IgG Antibodies to PV Antigens in CIN Patients and Age-Matched Controls†

Antigen	Positive cases n = 95	Positive controls n = 95	Odds ratio	95% CI	*P*-value*
E2-18	20 (21)	19 (20)	1.07	0.5–2.28	0.8574
E2-16	23 (24)	12 (13)	2.21	0.97–5.10	0.0039
L1-16	8 (8)	12 (13)	0.64	0.22–1.78	0.3443
VLP-16	43 (45)	19 (20)	3.31	1.66–6.65	0.0002
BPV-VLP	15 (16)	11 (12)	1.43	0.58–3.58	0.3984
VLP-16_{corr}	32 (34)	14 (15)	2.94	1.37–6.63	0.0022

†Values in parentheses are percent.
*χ^2 test for difference between two categories, CI = confidence interval.

TABLE II. Prevalence of IgG Antibodies to PV Antigens in Children*

Antigen	Number positive, n = 155	
	Mean + 2 SD	Mean + 3 SD
E2-18	29 (18.7)	23 (14.8)
E2-16	69 (44.5)	39 (25.1)
L1-16	31 (20)	28 (18.1)
VLP-16	7 (4.5)	5 (3.2)
BPV-VLP	8 (5.1)	6 (3.8)
VLP-16_{corr}	4 (2.5)	2 (1.3)

*Values in parentheses are percent.
Cut-off mean + 2SD or mean + 3SD

1:20 in 1% milk powder in PBS and left at room temperature for 120 min. Thereafter the procedure was as for the peptide ELISAs. To eliminate background caused by antibodies to baculovirus and tissue culture proteins which co-purify with the VLPs, the mean OD value of the serum on BPV-1 VLP (BPV-VLP)–coated wells was subtracted from the mean OD obtained on the wells coated with HPV-16 VLPs (VLP-16). These corrected results were designated VLP-16_{corr}.

Data Analysis

Data were analysed by χ^2 test using Epi Info Version 5 (Centers for Disease Control, Epidemiology Program Office, Atlanta, GA). In all tests the significance level for assessing deviations from the tested hypothesis was $P = .05$. The data were examined using two cut-off points for seropositivity. These were calculated using the mean of the control group values for each antigen (minus outliers) plus 2 standard deviations (mean + 2 SD) and the mean of the control group values (minus outliers) plus 3 SD (mean + 3 SD). Regression trendline analysis was by the least-squares method using Microsoft Excel.

RESULTS

The prevalence of IgG antibodies in women with CIN (patients) and age-matched female blood donors (controls) to the HPV-16 and HPV-18 peptides and the VLPs (using a cut-off, mean + 2 SD) is given in Table I. For each antigen the patients were compared to the controls to establish if there was any association of seropositivity with disease status. The seropositivity to E2-16 and VLP-16 was associated significantly with CIN ($P = .039$ and $P = .002$, respectively), whereas to BPV-VLP, L1-16, and E2-18 there was no association with CIN. For all antigens except E2-16 the OR did not change using either cut-off (mean + 2 SD or mean + 3 SD). Seropositivity to the E2-16 peptide using the cut-off of mean + 3 SD was threefold higher in CIN patients than controls (19% vs. 6%; $P = .008$; OR = 3.47). There was no correlation between the seropositivity to the different antigens for individual patients.

In our study a significant number of children had antibodies to HPV-16 and HPV-18 antigens (Table II). We chose to present our children's data with two ELISA cut-offs, i.e., mean + 3 SD and mean + 2 SD. The former values may result in some sera being scored as false-negatives. However, even when using these values a significant number of children were positive for HPV-16 E2 and L1 as well as HPV-18 E2 peptides. In contrast children's seropositivity to VLPs was low. No correlation was found between reactivities to the E2-16 and L1-16 peptides. Seropositivity was not related to the sex of the child.

Using the BPV-VLPs to eliminate background (VLP-16_{corr}) increased the specificity of the test but may have reduced the sensitivity of the test (Table I). However, there was little change in OR significance levels when comparing seropositivity to VLP-16 and the VLP-16_{corr} values (patients vs. controls).

Age Dependence of HPV-16 Seropositivity

Table III shows the age distribution of antibodies in adults to the E2-16, E2-18, and L1-16 peptides and the VLPs. The E2-16 antibody prevalence increased with age in CIN patients but dropped with age in the control group (Fig. 1a). The good degree of fit of the regression trendlines (Fig. 1a) was demonstrated by $R^2 = 0.9592$ for patients. For the controls the degree of fit of the trendline was not good ($R^2 = 0.6361$). The prevalence of antibodies to E2-16 amongst patients 40 years and younger (14/69) was less than amongst patients older than 40 years (9/26) with a relative risk (RR) of 1.71 ($P = .14$). The reverse was the case for VLP-16 (or VLP-16_{corr}), in which antibody prevalence was highest in the 20–29 age group in both patients and controls and decreased significantly with age for both patients and controls (Fig. 1b). The regression trendlines (Fig. 1b) demonstrated a good degree of fit for both patients ($R^2 = 0.9006$) and blood donors ($R^2 = 0.9571$). Prevalence to VLP-16 in the over 40 age group was lower (7/26) than in the under 40 age group (36/69) with RR = 1.94 ($P = .02$). There was no significant association with age for antibodies to the L1-16 peptide or the BPV-1 VLPs.

In the children's group the prevalence of antibodies to the E2-16 peptide decreased with age from 3 to 12 years (Table IV). Fewer children were seropositive in the 1–2 age group than in the 3–4 age group. Seropositivity to the L1-16 peptide showed no association with

TABLE III. Distribution of IgG Antibodies to HPV-16–Derived Peptides in Adults According to Age*

	Age in years							
	21–30, n = 24		31–40, n = 45		41–50, n = 18		>51, n = 8	
	Patient	Control	Patient	Control	Patient	Control	Patient	Control
E2-18	3 (12)	3 (12)	11 (24)	9 (20)	5 (28)	5 (28)	1 (12)	3 (38)
E2-16	3 (12)	3 (12)	11 (24)	7 (16)	6 (33)	2 (11)	3 (38)	0
L1-16	4 (17)	3 (12)	3 (7)	7 (16)	1 (6)	2 (11)	0	0
VLP-16	16 (67)	7 (29)	20 (44)	8 (18)	5 (28)	3 (17)	2 (25)	1 (12)
BPV-VLP	3 (12)	4 (17)	3 (7)	4 (9)	3 (17)	2 (11)	2 (25)	1 (12)
$VLP\text{-}16_{CORR}$	12 (50)	5 (21)	12 (27)	7 (16)	4 (22)	2 (11)	1 (12)	0

*Values in parentheses are percent.

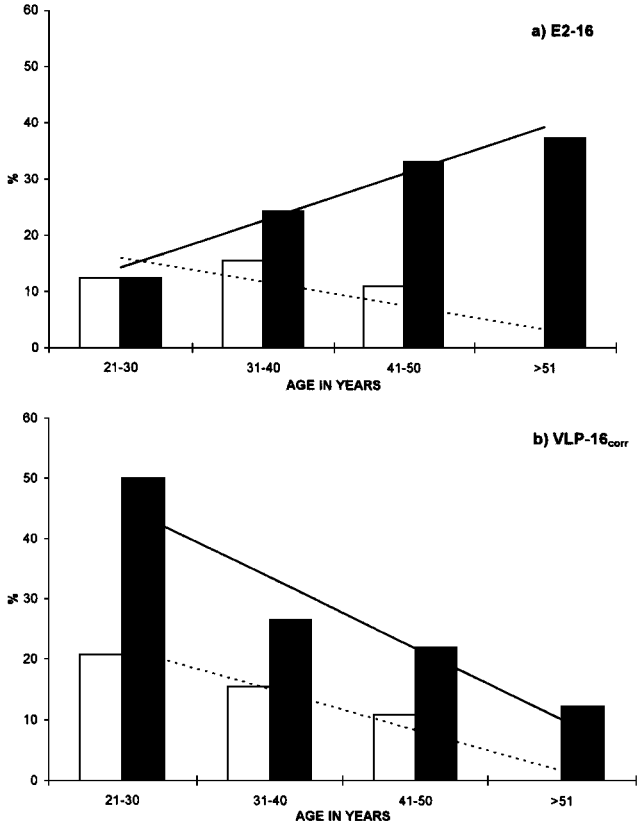

Fig. 1. Distribution of IgG antibodies to HPV-16 E2–derived peptide (**a**) and to $VLP\text{-}16_{corr}$ (**b**) in CIN patients (solid squares) and healthy controls (open squares) according to age. Trendlines a) E2-16: patients——$R^2 = 0.9592$, controls------$R^2 = 0.6361$; b) $VLP\text{-}16_{corr}$: patients——$R^2 = 0.9006$, controls------$R^2 = 0.9517$.

age. Amongst the children there were too few seropositive sera to look at the age distribution with the other antigens.

DISCUSSION

Our study shows a significantly higher incidence of antibodies to E2-16 and VLP-16 in women with CIN than in female blood donors. This is the first serological HPV study to be done on women from South Africa and confirms results from elsewhere. [Dillner, 1994; Kirnbauer et al., 1994; Nonnemacher et al., 1995]. However, the percentage of women with CIN who are seropositive to E2-16 or VLP-16 is lower than those reported previously [Dillner, 1994; Nonnemacher et al., 1995]. This is probably because of the different distribution of HPV types found in CIN in South Africa [Williamson et al., 1989]. The prevalence of VLP-16 antibodies in the controls was double that reported in Spain, which, unlike South Africa, is a country with a low incidence of cervical cancer [Nonnemacher et al., 1995].

None of the previous studies have analysed the age distribution of positive sera from women with CIN [Dillner et al., 1989; Nonnemacher et al., 1995; Dillner, 1994]. We have found that the occurrence of antibodies to E2-16 increases with age in women with CIN, whereas the converse is true in the age-matched blood donors. This indicates that antibodies to E2-16 may be a serological marker of CIN, but only in women over 40 years of age. Dillner et al. [1994] reported an increase in relative risk of seropositivity to E2-16 in women with cervical cancer over the age of 50 years. Unlike seropositivity to E2-16, the seropositivity to VLP-16 decreased with age in women with CIN. Therefore, antibodies to VLP-16 are only a serological marker of disease in younger women and are unlikely to prove a reliable marker of disease in older women as was suggested by Schiller and Roden [1995]. In women with normal cytology, the prevalence of HPV DNA has been shown to decrease with age [Melkert et al., 1993], which is similar to what is observed in the blood donor antibodies to E2-16 and VLP-16.

It is difficult to explain why the immune response to different HPV antigens in women with the same lesion should vary with age. The hormonal status of the women could influence HPV replication [Pater et al., 1994]. CIN in the younger women may be associated with a more recent viral infection, and there may be a large amount of virus being produced, which induces a good immune response. The older women may have had the lesion longer and are no longer shedding virus particles, but are still producing early HPV proteins. This hypothesis would be consistent with the integration of viral DNA in advanced disease, with the concomitant loss of late gene expression [zur Hausen, 1994]. It has been reported that antibodies to L1 are short lived unless there is a persistent infection [Wilkstrom et al.,

TABLE IV. Prevalence of IgG Antibodies to PV Antigens in Children According to Age*

	Age					
	1–2 years, n = 25	3–4 years, n = 31	5–6 years, n = 24	7–8 years, n = 27	9–10 years, n = 28	11–12 years, n = 20
E2-16	9 (36)	17 (55)	12 (50)	12 (44)	13 (46)	6 (20)
L1-16	6 (20)	4 (13)	7 (24)	2 (7.4)	6 (28)	6 (20)

*Values in parentheses are present.

1995], which implies that unless there is a productive HPV infection, L1 antibodies would decrease with time.

This is the first study reporting the prevalence of anti-E2-16 and E2-18 antibodies in children. There was a trend for the prevalence for antibodies to E2-16 to decrease with age, suggesting that children are infected early in life and therefore that the route of infection would be non-sexual. Some of the children may have been infected during birth. Cason et al. [1995] demonstrated high transmission rates from mother to infant, in which HPV-16 DNA was detected in 83% of cases.

As our control adults had a lower prevalence of antibody than the children, it is probable that the infections in children are not persistent. Children with antibodies to HPV-16 L1, L2, E4, E6, and E7 proteins have been reported in other studies [Cason et al., 1995; Muller et al., 1995]. It would be interesting to determine if mucosal immunity persists and whether adults that have been infected as children have a successful immune response to reinfection with HPV-16, resulting in fewer persistent infections. This information would have important implications for HPV vaccine strategies.

Our results show that few children have a IgG serological response to the antigens presented on VLP-16 and are more likely to have antibodies to E2-16. This may indicate a lack of sensitivity in the detection of VLP-16 antibodies or a lack of induction of IgG to VLP-16 in children. Since HPV E2 proteins are expressed in the lower layers of the epithelium and the L1 proteins are produced in the upper layers of the epithelium [Pfister and Fuchs, 1987], there is more likely to be an immune response to the early proteins. Alternatively most mucosal HPV infections in children are thought to be subclinical, and in such infections the amount of L1 protein made may be reduced.

The possibility of the children's seropositivity being false is unlikely as the results obtained with adult sera are similar to those reported elsewhere [Dillner et al., 1989; Nonnemacher et al., 1995; Dillner, 1994], confirming that the test was reliable. However the possibility of cross-reactivity with related antigens cannot be ruled out. Our study showed no correlation between the positive results obtained with the different HPV-16 antigens, which raises the question of whether the tests are specific for HPV-16. Alternatively different antibody responses may be detected at different stages of HPV infection. Nonnemacher et al. [1995] also reported no correlation between antibody responses to VLP-16 and HPV-16 E6 and E7.

There was no increase in seropositivity found to the E2-18 peptide in women with cervical disease compared with control women. This is consistent with results from our previous study which showed that HPV-18 DNA was rarely associated with cervical cancer in Cape Town [Williams et al., 1994] but was found in 3.5% of Cape Town women with normal cervical cytology [Ramesar et al., 1996].

In conclusion, this study shows that antibody response to E2-16 and VLP-16 are significantly associated with CIN. However, these associations are age dependent in adults, and a significant number of children have antibodies to E2-16 with no apparent disease. Therefore, age has to be considered in the search for serological markers of HPV-associated cervical disease. This study was done on relatively small number of sera and should be repeated with larger numbers, and also a wider range of cervical lesions should be examined. The high incidence of E2-16 antibodies in children suggests that a high proportion of children may be infected with HPV-16 or a related virus. HPV DNA isolation from children should help resolve some of the questions raised by these data.

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